Interaction between Peripheral and Central immune markers in Clinical High Risk for Psychosis

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1. Introduction

Several lines of evidence from epidemiological, preclinical and genetic studies suggest that abnormal immune response contribute to the pathophysiology of schizophrenia. Brain immune cells, in particular microglia and immunoproteins (e.g., cytokines and C-reactive protein, CRP), play an important role in neuroinflammatory responses both in the brain and periphery (Barron et al., 2017). In response to brain insults, microglial cells become activated, and at the molecular level increase the expression of a mitochondrial protein, translocator protein 18 kDa (TSPO). Currently, positron emission tomography (PET) using radioligands that target TSPO is the most reliable and valid method to quantify TSPO *in vivo* in brain (Hafizi et al., 2017a). Although previous studies using the prototypical radiotracer for TSPO, [^{11}C]PK11195, reported higher TSPO expression in schizophrenia (Doorduin et al., 2009; van Berckel et al., 2008), the majority of recent studies using second-generation radiotracers (e.g., [^{11}C]DPA-713, [^{11}C]PBR28, and [^{18}F]FEPPA) and validated outcome measures found reduced (Collste et al., 2017) or no significant difference (Hafizi et al., 2017a, 2018a; Coughlin et al., 2016) in brain TSPO expression in first-episode psychosis and individuals at clinical high risk for psychosis (CHR) (Hafizi et al., 2017b) as compared to healthy controls. Moreover, a recent meta-analysis (Plavén-Sigray et al., 2021) reported a significant decrease in TSPO expression in schizophrenia.

CRP and cytokines are commonly used as markers of inflammatory status. Based on their function, cytokines can be categorized as either pro-inflammatory such as Interleukin (IL) 1 beta (IL-1β), IL-6 and IL-12, or anti-inflammatory such as IL-2 and IL-10. A meta-analysis (Miller et al., 2011) combining data from 40 studies identified increases in peripheral pro-inflammatory and decreases in anti-inflammatory cytokine proteins in schizophrenia. The dynamic balance between these two categories of cytokines contributes to proper immune response.

CRP is an acute phase protein which is produced and released by liver cells in response to inflammation. Several studies have reported elevated levels of CRP in schizophrenia (Miller et al., 2014; Fernandes et al., 2016; North et al., 2021). Supporting this, a longitudinal study has reported an association between adolescent CRP level and consequent schizophrenia diagnosis by age 27 (odds ratio = 1.25) (Metcalf et al., 2017). Studies on cytokines levels in blood and cerebrospinal fluid (CSF) revealed elevated expressions of IL-1β, IL-6, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-12 and transforming growth factor beta (TGF-β) in blood and IL-1β in CSF of first-episode psychosis patients (Miller et al., 2011). A population-based longitudinal study reported an association between childhood IL-6 levels and risk of psychosis in adulthood (Khandaker et al., 2014). Similarly, pro-inflammatory cytokine levels (IL-6) were elevated in CHR, and associated with severity of attenuated psychotic symptoms (Stojanovic et al., 2014). More recently, plasma-based markers of inflammation were found to be predictors of conversion to a full psychotic break in CHR (Perkins et al., 2015). Several cross-sectional and post-mortem studies have also found large increases in cortical and subcortical cytokine levels in about half of those with chronic schizophrenia (Miller

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et al., 2011; North et al., 2021; van Kesteren et al., 2017; Boerrigter et al., 2017; Fillman et al., 2013, 2016); however, a post-mortem meta-analysis combining 41 studies reported no consistent diagnostic group effect on cytokine levels in schizophrenia samples (van Kesteren et al., 2017). Importantly, an inflammatory stratification approach was not applied in most studies which hinders their interpretation. Interestingly, previously reported studies in schizophrenia (Boerrigter et al., 2017) revealed that a subgroup of about ~40% of people with schizophrenia had a pattern of relatively elevated cytokine expression compared to 20% in the peripheral blood (Fillman et al., 2016) and 10% in the post-mortem prefrontal cortex tissue (Fillman et al., 2013) in healthy controls. However, to our knowledge, similar inflammatory subsets have never been studied in individuals at risk for psychosis such as the well characterized CHR state (Hafizi et al., 2017b).

To date, only one *in vivo* study (Coughlin et al., 2016) has investigated the association between markers of inflammation in the brain (TSPO) and peripheral tissue (plasma) in schizophrenia, and reported no relationship, similar to the post-mortem brain when not stratified by inflammatory status. In the present study, we measured peripheral inflammatory protein levels together with TSPO expression in the brain (with [18F] FEPPA, or fluorine F 18–labeled N-(2-(2-fluoroethoxy)benzyl)-N-(4-phenoxypyridin-3-yl)acetamide using full kinetic modelling in a High-Resolution Research Tomograph (HRRT)) in a relatively large sample of mostly unmedicated CHR and healthy controls. We primarily aimed to (Barron et al., 2017) test study group (CHR and Healthy controls) differences in peripheral inflammatory markers and test for any associations with symptom and cognition measures, (Hafizi et al., 2017a) identify clusters in the entire cohort and CHR group alone based on their peripheral inflammatory (including IL-2 and IL-10) profile, (Doorduin et al., 2009) examine cluster differences in the dorsolateral prefrontal cortex and hippocampal TSPO levels (Kreisl et al., 2013; Busse et al., 2012). Further, in the exploratory analyses, we aimed to (Barron et al., 2017) examine cluster differences for symptom severity and cognition and (Hafizi et al., 2017a) identify the independent peripheral predictors of brain TSPO expression in the combined cohort. We expected to identify a subset of people with elevated inflammatory state (larger in the CHR cohort). We hypothesised that this inflammatory subset would be significantly associated with [18F]FEPPA total volume of distribution (V_T) binding and have worse symptomatology and lower cognition. Finally, we expected to identify peripheral inflammatory markers that may be predictive of TSPO V_T.

2. Materials and methods

2.1. Participants

Fifty-nine participants including 39 CHR and 20 healthy controls were included in this study. Most of the individuals in the CHR group were antipsychotic-naive (n = 33). Two healthy controls and one CHR whose PET images were of insufficient quality were excluded from the PET analyses, and another healthy control was excluded due to low-affinity binder (LAB) genotype which cannot be reliably quantified with [18F] FEPPA. While CRP levels were available for all participants, the cytokine levels were only available for 38 CHR (Age, 20.95 ± 2.78 (Mean ± Standard deviation (SD)) and 20 healthy controls (Age, 21.30 ± 2.06 (Mean ± SD) (see Table 1). The study was performed under a repository protocol that allowed a re-analysis of previously acquired data approved by the Centre for Addiction and Mental Health Research Ethics Board and now approved under Clinical and Translational Sciences (CaTS) BioBank by Research Ethics Board (REB) of the Centre intégré universitaire de santé et de services sociaux (CIUSSS) de l'Ouest-de-l'Île-de-Montréal – Mental Health and Neuroscience subcommittee. Participants were recruited at the Centre for Addiction and Mental Health (CAMH) from January 1, 2015, to October 20, 2018 (Ontario, Canada). The study was performed in accordance with Good Clinical Practice guidelines, regulatory requirements, and the Code of Ethics of

Table 1
Demographics and characteristics of the participants.

		Healthy controls (n = 20)	Clinical high-risk (n = 38)	*t*-Test or χ^2 Test value	*p* value
Age (years), Mean ± SD		21.30 ± 2.06	20.95 ± 2.78	t = 0.50	*p* = 0.62
Sex	Male, n	7	21	χ^2 = 2.16	*p* = 0.14
	Female, n	13	17		
BMI (kg/m²), Mean ± SD		23.25 ± 4.79	23.63 ± 4.98	t = - 0.28	*p* = 0.78
TSPO rs6971 Genotype[a]	HAB, n	14	24	χ^2 = 0.63	*p* = 0.43
	MAB, n	5	14		
PET Parameters, Mean ± SD[a]	Specific activity (mCi/μmol)	1996.10 ± 1908.94	1678.42 ± 1305.83	t = 0.73	*p* = 0.47
	Mass injected (μg)	1.70 ± 1.35	1.86 ± 1.49	t = −0.39	*p* = 0.70
	Amount injected (mCi)	4.96 ± 0.34	5.10 ± 0.26	t = −1.68	*p* = 0.10
Tobacco use	Non-users, n	20	31	χ^2 = 4.19	**p = 0.04**
	Users, n	0	7		
Cannabis use[b]	Non-users, n	20	34	χ^2 = 2.26	*p* = 0.13
	Users, n	0	4		

Abbreviations: BMI – body mass index; χ^2 – Chi-squared statistic; HAB – high-affinity binder; μg – microgram; μmol – micromole; mCi – millicurie; MAB – mixed-affinity binder; PET - positron emission tomography; *p* value - significance; SD – Standard deviation; TSPO - Translocator protein 18 kDa.
[a] Two healthy controls and one CHR were excluded from the positron emission tomography (PET) analyses due to unreliable PET data, and another healthy control was excluded due to low-affinity binder (LAB) genotype which cannot be quantified with [18F]FEPPA.
[b] 4 CHR individuals had a positive urine drug screen for cannabis.

the World Medical Association (Declaration of Helsinki). Written informed consent was initially obtained from all participants at the beginning of screening after a full explanation of anticipated study procedures. The majority of the participants (healthy controls, n = 20 and CHR, n = 35) in this study were also part of our previous studies (Hafizi et al., 2017b, 2018a, 2018b).

To be eligible, CHR individuals had to meet the following criteria: fulfilment of diagnostic criteria for prodromal syndrome as per the Criteria of Prodromal Syndromes (COPS) (Miller et al., 2002) with no current axis I disorders such as depression which has been identified to be associated with microglial activation (Setiawan et al., 2015), as determined with the Structured Clinical Interview for DSM-IV-TR (SCID) (First and Gibbon, 2004). Healthy controls did not have any history of psychiatric illness, psychoactive drug use, and/or first-degree relative with a major mental illness. Participants were excluded for any of the following: clinically significant medical illness, inflammatory condition that warranted anti-inflammatory medication use and/or chronic anti-inflammatory medication use, elevated body temperature indicating possible infection/inflammatory status, pregnancy or current breastfeeding, presence of metal implants precluding MRI scan. In CHR, clinical status and severity of symptoms (e.g., psychosis-risk symptoms) were assessed with the Structured Interview for Psychosis-Risk Syndromes (SIPS), Scale of Psychosis-Risk Symptoms (SOPS) (Miller et al., 2002), Calgary Depression Scale (CDS) (Addington et al., 2014), Snaith-Hamilton Pleasure Scale (SHAPS) (Snaith et al., 1995), and Apathy Evaluation Scale (AES) (Marin et al., 1991). Neurocognitive performance was assessed using the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (Randolph et al., 1998).

2.2. PET and structural MRI data acquisition and analysis

PET and MRI data acquisition have been described in detail elsewhere (Hafizi et al., 2018a; Kenk et al., 2015) and are summarized below. A proton density-weighted (PD) brain MRI scan was obtained for

each subject using a 3T MR-750 scanner (General Electric Medical Systems). All [18F]FEPPA PET scans were performed using a high-resolution neuro-PET camera system (HRRT, Siemens Molecular Imaging, Knoxville, TN, USA) for 125 minutes following an intravenous bolus injection of 187.11 ± 10.83 MBq of [18F]FEPPA. Arterial blood samples were collected automatically using an automatic blood sampling system (Model PBS-101, Veenstra Instrument, Joure, Netherland) for the first 22.5 minutes after radioligand injection at a rate of 2.5 mL/min and manually at −5, 2.5, 7, 12, 15, 20, 30, 45, 60, 90, and 120 min to measure radioactivity in blood and determine the relative proportion of radiolabelled metabolites. Dispersion-and metabolite-corrected plasma input function was generated as previously described (Rusjan et al., 2011).

2.3. PET image processing and calculation of total volume of distribution (V_T)

Time-activity curves were extracted for the dorsolateral prefrontal cortex (DLPFC) and hippocampus using validated in-house imaging pipeline ROMI (Rusjan et al., 2006). DLPFC and hippocampus were chosen as the prioritized regions of interest (ROI) based on the previous data (Kreisl et al., 2013; Busse et al., 2012). The ROI was delineated using individual PD MRI. Kinetic parameters of [18F]FEPPA were derived from the time-activity curves using the two-tissue compartment model (2-TCM) and plasma input function to obtain the V_T for each region of interest, which has been validated for [18F]FEPPA quantification and described elsewhere (Kenk et al., 2015; Rusjan et al., 2011).

2.4. TSPO rs6971 polymorphism genotyping

Participants were genotyped for their TSPO rs6971 polymorphism and categorized as high-(HAB), mixed- (MAB), or LAB, as described elsewhere (Mizrahi et al., 2012).

2.5. Serum acquisition and measurement of high sensitive-CRP (HsCRP) and cytokines

The serum samples obtained on the day of the PET scan from early non-fasting (period since last caloric intake, 0–2.9h) participants were isolated from whole-blood specimens collected in BD Vacutainer K2EDTA tubes. The samples were centrifuged at 3000 r/min for 15 min at 4 °C and was then transferred to a fresh polypropylene microtube (1.5 mL capacity) which was stored at −80 °C. No samples were thawed more than once before analysis. Cytokine levels in serum were measured with the MILLIPLEX Multi-Analyte Profiling Human Cytokine/Chemokine Assay employing Luminex technology according to manufacturer's protocol. The cytokines included in the assay were IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ. HsCRP was measured in serum using a high-sensitivity ELISA according to the manufacturer's instructions (IBL-international, Hamburg, Germany). We quantitatively determined the steady state level of the circulating inflammatory (both pro- and anti-inflammatory) cytokines from serum using the MILLIPLEX Multi-Analyte Profiling (MAP) Human Cytokine/Chemokine Kit for 96-well assay (Millipore) run on a Luminex platform (MXH8060-2). 25 μl of neat serum from each participant was serially diluted and was diluted 1:4 using reconstituted stock standards supplied by the manufacturer. A standard curve range of 3.2–10,000 pg/mL was used to calculate average values generated using Millipore Analyst Software. A low and a high concentration quality control (QC) supplied by the manufacturer for each analyte was used to calibrate and quantify the analyte concentrations. For quality assurance, each sample was run twice, and the mean derived for each sample was used as the index value. Additionally, two kit-supplied quality controls were run on each plate in duplicate and confirmed to fall within the expected range (see S.1 for detailed description).

2.6. Statistical analyses

We used chi-square (χ^2) tests and independent sample t-tests to evaluate differences in categorical variables (e.g., sex and TSPO rs6971) and in continuous variables (e.g., age), respectively between CHR and healthy controls. Initially, we tested for study group by cytokine interaction i.e., the effect of group which varies between cytokines using random effects mixed model analysis, adjusted for body mass index (BMI) with cytokines (interleukins and CRP) as a repeated within-subject fixed factor. Further, interleukin and CRP levels were compared using the Mann–Whitney *U* test and their mean ranks were presented. The results of the analyses are reported including the extreme outliers (defined as inflammatory marker values outside 3rd quartile + 3*interquartile and 1st quartile – 3*interquartile ranges or as determined using box-plot analysis) given their potential importance in understanding the divergence of study groups based on the actual measured peripheral inflammatory marker profile (see supplementary S.2 for the analysis excluding these outliers and S.3 for the analyses including only pro-inflammatory markers (excluding IL-2 and IL-10)). The extreme outliers observed study group-wise include CRP (HC, n = 1; CHR, n = 6), IFN-γ (HC, n = 1), IL-12 (CHR, n = 2) and IL-2 (HC, n = 1). We also explored the associations between the differentially expressed peripheral inflammatory biomarker(s) and clinical symptom scales (SOPS and CDS (CHR), cognition and other behavioural measures such as anhedonia, apathy) in the respective study group by using Pearson partial correlations, controlling for BMI.

Further, the inflammatory cytokine values were tested for normality (Shapiro-Wilk) and homogeneity of variances (Levene's test). Given the low-skewed distribution, all inflammatory cytokine values were log transformed to approximately conform to normality. Any extreme outlier values for cytokines (IL-12: HC, n = 1) following log transformation were excluded from the following cluster and exploratory analyses.

To identify inflammatory clusters based on peripheral inflammatory marker levels, a recursive two-step cluster analysis was performed on the entire cohort and then on the CHR cohort alone as previously described (Boerrigter et al., 2017; Fillman et al., 2016). The clustering for the entire cohort was performed in 57 individuals (19 HC and 38 CHR) (excluding extreme outlier, IL-12: HC, n = 1). Briefly, the clustering algorithm was run once with all predictive factors, in this case, all nine inflammatory markers (CRP, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ). The model developed was examined for silhouette measure of cohesion and separation and the contribution of each variable to the model. The overall model quality was required to be >0.5, with predictors of least importance removed until all predictors had significant contribution to the model (≥0.5 on a scale of 0-1.0) (Bacher et al., 2004). Following the clustering of the entire cohort, [18F]FEPPA V_T differences were tested using random effects mixed model analysis in prioritized ROIs (DLPFC and hippocampus), controlling for TSPO rs6971 polymorphism and BMI. Non-significant interactions were removed from the model. Further, inflammatory cluster by subscale interactions i. e., effect of cluster which varies between subtests in attenuated psychosis symptom (SOPS positive, negative, disorganization, general) and cognition (RBANS immediate memory, visuospatial ability, language, attention, delayed memory) subscales were explored using random effects mixed model analysis with SOPS subscales and RBANS subtests as repeated within-subject fixed factor, respectively adjusted for BMI. Univariate analysis of variance was used for assessing inflammatory cluster differences in SOPS total symptom severity scale (CHR), RBANS total scale and other behavioural measures (depression (CHR), anhedonia, apathy) as exploratory analyses. Same procedures were followed for the CHR group (see Fig. 1 for the schematic illustration of the analyses).

Finally, we investigated peripheral markers that predict [18F]FEPPA V_T in the brain. We used a backward random effects mixed model analysis to find the independent predictors of [18F]FEPPA V_T in the
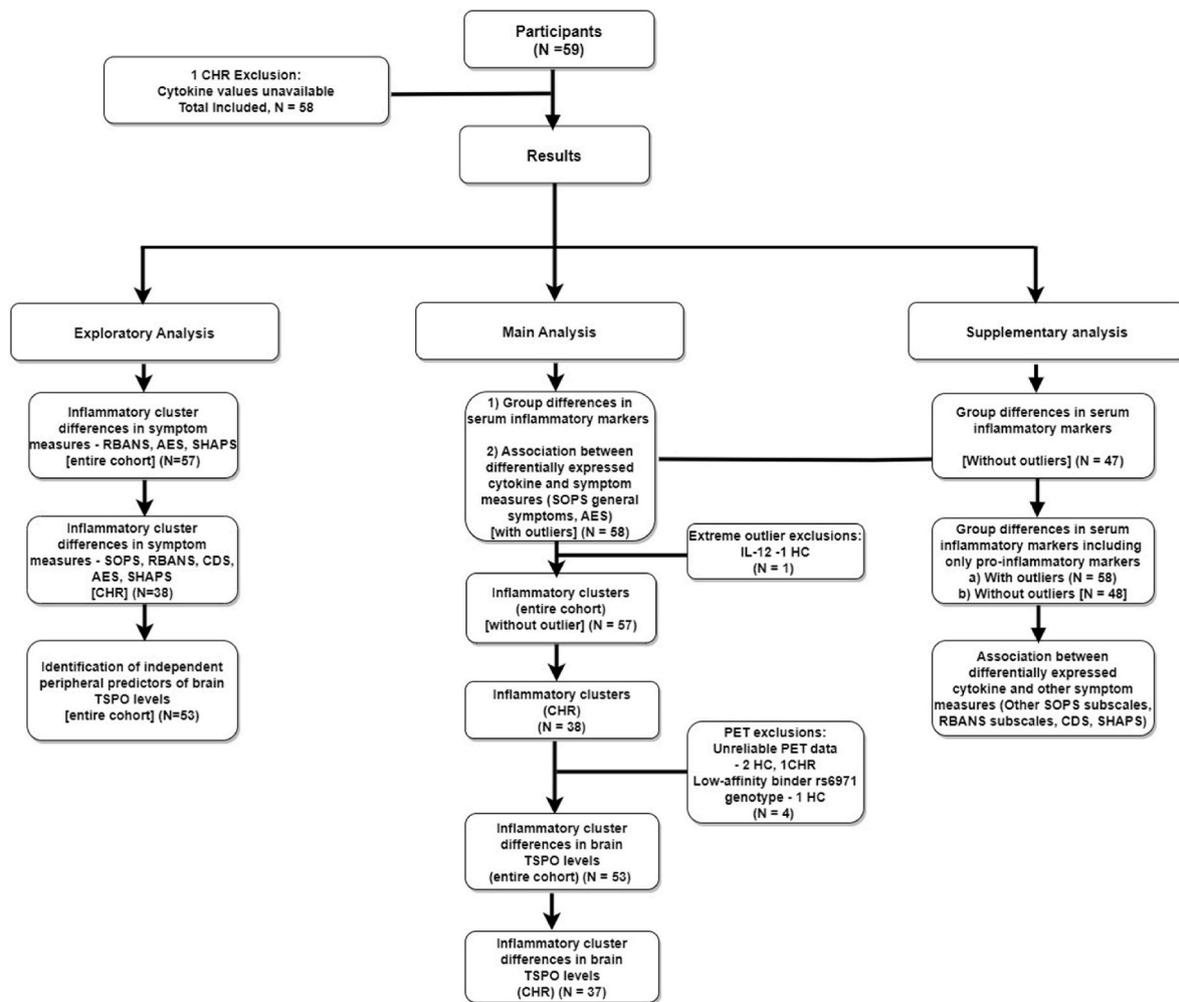
Fig. 1. The schematic CONSORT diagram illustrates the analyses included in the study.

Abbreviations: AES - Apathy Evaluation Scale; CDS - Calgary Depression Scale; CHR – Clinical High-risk; HC – Healthy control; IL-12 – Interleukin-12; N – Sample size; PET – Positron emission tomography; RBANS - Repeatable Battery for the Assessment of Neuropsychological Status; SOPS - Scale of Psychosis-Risk Symptoms; SHAPS Snaith-Hamilton Pleasure Scale; TSPO - Translocator protein 18 kDa.

whole sample by including inflammatory serum proteins as covariates, controlling for TSPO rs6971 polymorphism, clinical group and BMI. Brain regions (DLPFC and hippocampus) were entered as within-subject factors. We started by including all the variables and dropping the variable with the least significance manually, until all the variables in the model were significant.

Statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA). The significance level was set at p<0.05 two-tailed.

Fig. 1. Schematic CONSORT diagram as per analysis plan.

3. Results

3.1. Demographic and clinical characteristics

Demographic and clinical characteristics of the participants are presented in Table 1. There were no significant group differences in age, sex, TSPO rs6971 genotype, PET parameters (specific activity, mass injected, and amount injected), and cannabis use. There was a significant group effect for tobacco use.

3.2. Study group differences in serum inflammatory markers and association with symptom measures

#### 3.2.1. Group differences in peripheral serum inflammatory marker levels

We found a statistically significant study group-by-cytokine interaction ($F_{(17,107.69)} = 53.46$, $p < 0.001$), controlling for BMI ($F_{(1,56.77)} = 0.63$, $p = 0.43$). We found that IL-8 levels in the CHR group (mean rank $= 33.14$) were significantly higher than the healthy control group (mean rank $= 22.58$) ($U = 241.500$, $p = 0.02$ (see supplementary S.4.1 for mean ranks) with pairwise comparisons surviving corrections for multiple testing (Fig. 2).

Fig. 2. Serum inflammatory marker concentrations between study groups.

3.2.2. Positive association between IL-8 levels and general symptom severity and apathy in CHR

Within the CHR group, increased IL-8 levels were positively correlated with SOPS general symptom score ($r = 0.43$, $p = 0.008$, Fig. 3A), surviving corrections for number of SOPS subscales. IL-8 levels also showed a trend for positive association with apathy ($r = 0.32$, $p = 0.059$, Fig. 3B) (see supplementary S.4.2 for other scales tested) in individuals at CHR for psychosis.

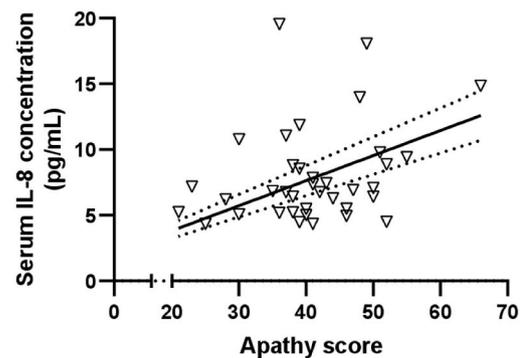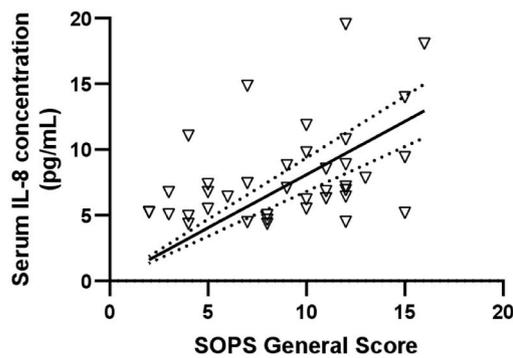Fig. 3. Association between IL-8 levels and prodromal general symptom severity and Apathy in CHR.
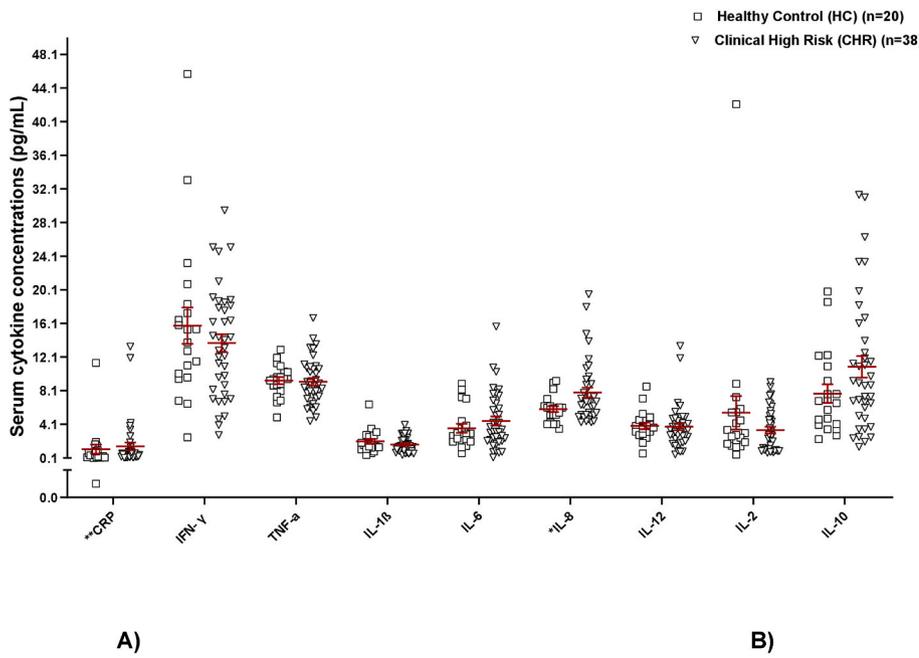
Fig. 3. Relationship between IL-8 and **A)** General symptom severity score and **B)** Apathy score as measured by Scale of Psychosis-Risk Symptoms (SOPS) General symptom subscale and Apathy Evaluation Scale (AES), respectively, in CHR individuals, adjusted for BMI.
Abbreviations: BMI – body mass index; CHR – clinical high-risk; IL-8 – Interleukin-8; mL – milliliter; pg - picograms.

3.3. Identification of inflammatory clusters

3.3.1. Inflammatory clusters in the entire cohort

Prior to cluster analysis, one extreme outlier for inflammatory marker, IL-12 (HC, n = 1) following log-transformation was noted. Analysis of the data excluding this outlier yielded three clusters in the entire cohort characterized by inflammatory markers IL-1β, IL-2 and IFN-γ. The clusters had an overall model quality (Silhouette measure) of 0.5 and all three variables (IL-1β, IL-2 and IFN-γ in order of their contribution) contributed significantly to the model (≥0.5 on a scale from 0.1 to 1.0). The clusters were labeled 'low cytokine' (total n = 16; 13 CHR and 3 healthy controls), 'intermediate cytokine' (total n = 21; 12 CHR and 9 healthy controls) and elevated cytokine' (total n = 20; 13 CHR and 7 healthy controls) clusters (Fig. 4A).

3.3.2. Inflammatory clusters in the CHR group

Two clusters characterized by inflammatory markers IL-2, IL-1β and IFN-γ were identified. The resulting two clusters had an overall model quality (Silhouette measure) of 0.5 and were comparable to the clusters identified in the whole cohort except for the order of their contribution (IL-2, IL-1β and IFN-γ in order of their contribution) to the model (≥0.5 on a scale from 0.1 to 1.0). The clusters were labeled 'elevated cytokine' (total n = 24) and 'low cytokine' (total n = 14) (Fig. 4B).

Fig. 4. Inflammatory clusters based on peripheral inflammatory marker levels.

3.4. Inflammatory cluster differences in brain TSPO levels

3.4.1. TSPO levels were not different between inflammatory clusters in the entire cohort

We found no significant differences in [^{18}F]FEPPA V_T between inflammatory clusters (main cluster effect: F (Hafizi et al., 2017a; Takano et al., 2010) = 2.07, p = 0.14; ROI effect: F (Barron et al., 2017; Takano et al., 2010) = 5.16, p = 0.03, controlling for TSPO rs6971 polymorphism and BMI effect (F (Barron et al., 2017; Takano et al., 2010) = 7.33, p = 0.009) in the entire cohort. [^{18}F]FEPPA V_T levels were relatively same among the different inflammatory clusters namely, low (mean: 9.74; 95% CI: 8.15 to 11.32), intermediate (mean: 8.62; 95% CI: 7.36 to 9.88) and elevated (mean: 10.50; 95% CI: 9.17 to 11.83) cytokine clusters (Fig. 5A).

In the exploratory analysis, we found no main effect of inflammatory clusters on RBANS total score (F (Hafizi et al., 2017a; Dawidowski et al., 2021) = 1.39, p = 0.26) or RBANS subtest scores (immediate memory, visuospatial ability, language, attention, delayed memory) (F$_{(2,60.71)}$ = 1.35, p = 0.27). We also did not find any significant inflammatory clusters-by-RBANS subscales interaction (F$_{(8,95.79)}$ = 1.78, p = 0.09),
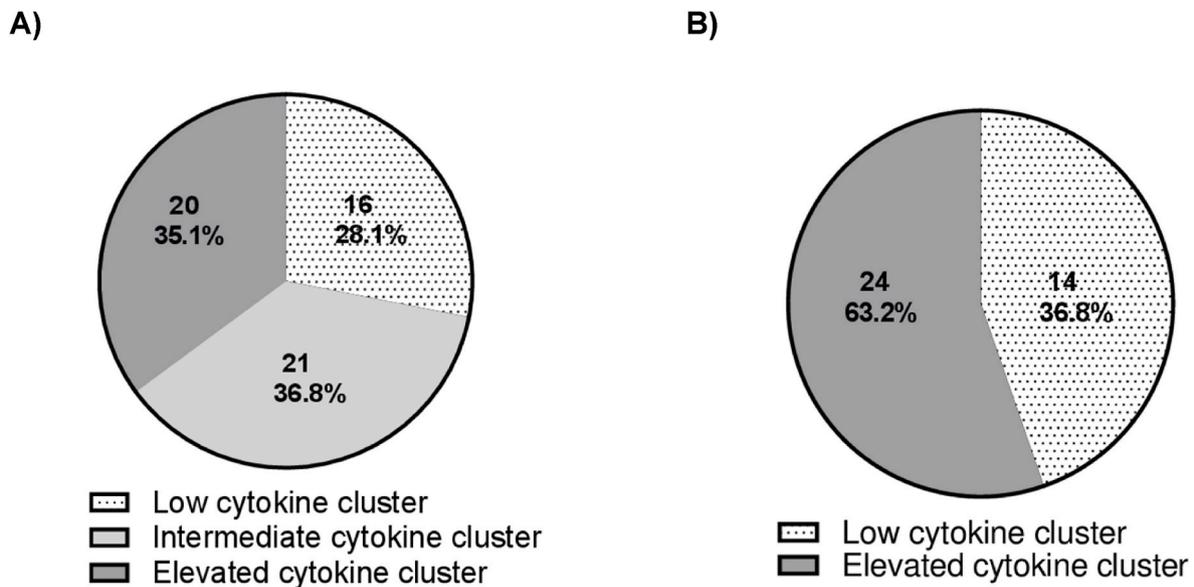
Fig. 4. Inflammatory clusters of **(A)** entire cohort characterized by low, intermediate, and elevated inflammatory marker levels and **(B)** CHR group characterized by low and elevated inflammatory marker levels in serum are represented.
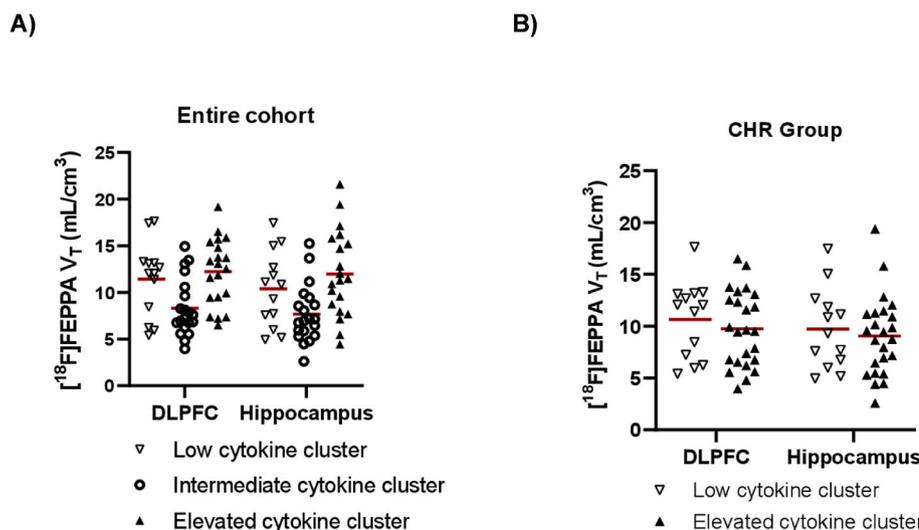

Fig. 5. The scatter dot plot graph reflects [^{18}F]FEPPA regional binding V_T in prioritized ROIs (i.e., DLPFC and hippocampus) among the three inflammatory clusters (low, intermediate and elevated cytokine cluster) in the entire cohort **(A)** and two inflammatory clusters (low and elevated cytokine cluster) in the CHR group **(B)**. Horizontal bar indicates group mean adjusted for TSPO rs6971 genotype and BMI using the estimated marginal means of each region.

Abbreviations: CHR - Clinical High Risk; DLPFC - dorsolateral prefrontal cortex; [^{18}F]FEPPA - fluorine F 18–labeled N-(2-(2-fluoroethoxy)benzyl)-N-(4-phenoxypyridin-3-yl)acetamide; mL/cm^3 – milliliter/cubic centimetre; V_T - total volume of distribution.

adjusted for RBANS subscales and BMI effect. There were also no significant differences in clinical symptom measures (i.e., apathy (F (Hafizi et al., 2017a; Dahan et al., 2018) = 0.95, p = 0.39), anhedonia (F (Hafizi et al., 2017a; Dawidowski et al., 2021) = 1.73, p = 0.19) among inflammatory clusters, controlling for BMI.

3.4.2. TSPO levels were not different between inflammatory clusters in the CHR group

There were no significant differences in [^{18}F]FEPPA V_T between inflammatory CHR clusters (main cluster effect: F (Barron et al., 2017; Park and Miller, 2020) = 0.003, p = 0.95; ROI effect: F (Barron et al., 2017; Park and Miller, 2020) = 7.24, p = 0.01, controlling for TSPO rs6971 polymorphism and BMI effect (F (Barron et al., 2017; Park and Miller, 2020) = 2.00, p = 0.17) (Fig. 5B).

In the exploratory analysis, no difference was observed between inflammatory CHR clusters in attenuated psychosis symptoms (SOPS total symptoms severity score (F (Barron et al., 2017; Mizrahi et al., 2012) = 0.56, p = 0.46) or individual symptom dimensions (positive, negative, disorganization, general): F$_{(1,50.13)}$ = 0.32, p = 0.58) or found any

significant inflammatory group-by-SOPS-subscale interaction (F$_{(3,66.81)}$ = 0.49, p = 0.69). We also found no statistically significant main effect of inflammatory clusters on RBANS total score (F (Barron et al., 2017; Mizrahi et al., 2012) = 3.76, p = 0.06) or RBANS subscale scores (F$_{(1,42.92)}$ = 3.49, p = 0.07). We also did not find any significant inflammatory clusters-by-RBANS subscale interaction (F$_{(4,73.68)}$ = 1.85, p = 0.13), adjusted for RBANS subscales and BMI effect. There were also no significant differences in other clinical symptom measures (i.e., depression (F (Barron et al., 2017; Mizrahi et al., 2012) = 0.10, p = 0.76), apathy (F (Barron et al., 2017; Rusjan et al., 2006) = 0.02, p = 0.89) and anhedonia (F (Barron et al., 2017; Mizrahi et al., 2012) = 0.18, p = 0.67)) among inflammatory clusters, controlling for BMI.

Fig. 5. [^{18}F]FEPPA (V_T) in the prioritized ROIs among inflammatory clusters in the entire cohort and CHR group.

3.5. CRP, IL-1β, TNF-α and IFN-γ are the independent predictors of brain TSPO expression

CRP, IL-1β, TNF-α and IFN-γ levels were significantly associated

independently with [^{18}F]FEPPA V_T in the combined cohort (CRP: F (Barron et al., 2017; Takano et al., 2010) = 28.48, p < 0.001; IL-1β: F (Barron et al., 2017; Takano et al., 2010) = 12.51, p = 0.001; TNF-α: F (Barron et al., 2017; Takano et al., 2010) = 7.73, p = 0.008; IFN-γ: F (Barron et al., 2017; Takano et al., 2010) = 4.39, p = 0.04) controlling for TSPO rs6971 polymorphism, clinical group, ROI and BMI effect (BMI effect: F (Barron et al., 2017; Takano et al., 2010) = 0.62, p = 0.43). We found no significant group interactions in the model (Group*CRP: F (Barron et al., 2017; Takano et al., 2010) = 1.64, p = 0.21; Group* IL-1β: F (Barron et al., 2017; Takano et al., 2010) = 2.69, p = 0.11; Group* TNF-α: F (Barron et al., 2017; Takano et al., 2010) = 0.81, p = 0.37; Group*IFN-γ: F (Barron et al., 2017; Takano et al., 2010) = 1.17, p = 0.29).

4. Discussion

Here, we report increased IL-8 levels that show a significant positive association with prodromal general symptom severity and a trend-level association with apathy in individuals at CHR for psychosis. We identified three distinct clusters with varying grades of inflammation (low, intermediate, and elevated) characterized by markers IL-1β, IL-2 and IFN-γ in the combined cohort and two clusters (low and elevated) in the CHR group. Brain TSPO levels were not different between the inflammatory clusters (entire cohort or CHR group). Finally, we show that CRP, IL-1β, TNF-α and IFN-γ levels are the independent peripheral predictors of TSPO expression in the brain.

IL-8, a potent chemokine was found to be elevated by 33.6% in CHR individuals when compared to healthy controls. In contrast, previous studies in CHR and Ultra-high risk (UHR) individuals found unaltered peripheral IL-8 levels (Park and Miller, 2020; Misiak et al., 2021). However, meta-analyses in first episode psychosis antipsychotic-naive (Miller et al., 2011; Çakici et al., 2020) and acute relapsed chronic patients (Goldsmith et al., 2016; Wang and Miller, 2018) repeatedly showed elevated peripheral and CSF (Gallego et al., 2018) IL-8 levels similar to our findings (but see (Pillinger et al., 2019)). Additionally, previous data suggests absence of elevated IL-8 levels in medicated patients (Frydecka et al., 2018). In our sample, of the 38 CHR individuals, only 5 of them were on low-dose antipsychotic treatment and others were antipsychotic-naive which may explain the findings. However, another meta-analysis non-stratified by treatment or patient cohort found IL-8 levels to be unaffected in antipsychotic treated patients (Romeo et al., 2018).

Research on chemokine alterations in schizophrenia is limited (Frydecka et al., 2018) in comparison to other non-chemokine interleukins (Goldsmith et al., 2016; Capuzzi et al., 2017) however, an interesting study on pregnant women reported a correlation between increased serum IL-8 levels and psychosis-risk in the adult offspring (Brown et al., 2004). Additionally, in our CHR sample, serum IL-8 levels also showed a positive correlation with general symptom severity and a trend-level association with apathy. These outcomes are consistent with our previous findings in CHR (Da Silva et al., 2018) demonstrating a positive relationship between lactate levels and prodromal negative symptoms. Similar results were also observed in a study investigating diagnostic markers in CHR (North American Prodrome Longitudinal Study (NAPLS)). The study showed that the blood levels of several interleukins including IL-8 were positively correlated with the severity of positive symptoms (i.e., delusional ideas), poor attention, and dysphoric mood (characteristic of general symptom severity) (Perkins et al., 2015). Previous studies in schizophrenia also reported elevated IL-8 levels in patients with aggravated negative symptoms (Enache et al., 2021) and clinical global impression (CGI) severity (Dahan et al., 2018) similar to positive correlation with apathy observed in our CHR sample.

We identified three distinct clusters (IL-1β, IL-2 and IFN-γ) with differentially expressed inflammation levels (low, intermediate and elevated) in the entire cohort which were comparable to the inflammatory cluster composition in the CHR group (IL-2, IL-1β and IFN-γ)

characterizing the two clusters (low and elevated) identified. Elevations in the individual peripheral inflammatory markers identified in our inflammatory subsets (characterized by IL-1β, IL-2 and IFN-γ) have been reported in patients with psychosis extensively (Dawidowski et al., 2021). Whether this represents an inflammatory process defined by individual or subgroups of markers is still unclear. Our study takes the first step in identifying inflammatory subgroups within mostly unmedicated CHR and assesses their association with microglial activation. Previously, a study in psychosis population (Lizano et al., 2021) found that elevated levels of TNFα, CRP, VEGF and IL-6 were associated with changes in regional brain volume in multiple brain regions including hippocampus. However, in our study sample, brain TSPO levels were not differentially expressed between inflammatory clusters (entire cohort or CHR). This finding is consistent with six previous PET studies of TSPO expression in psychosis using second-generation radioligands and validated two-tissue compartment model for TSPO, including CHR (Hafizi et al., 2017b), first-episode psychosis (Coughlin et al., 2016; Hafizi et al., 2018a) or chronic schizophrenia (Kenk et al., 2015; Takano et al., 2010) that reported no significant differences in microglial activation between study groups.

Previous studies have shown that knockdown of TSPO influenced aggravated inflammatory response to lipopolysaccharide (LPS), a potent neuroimmune stimulus while overexpression of TSPO caused reduced cytokine production (Wang et al., 2016; Bae et al., 2014) implying a negative regulatory mechanism (Bae et al., 2014; Ma et al., 2016). In our study sample, almost all the parameters are normal which may explain the negative results. Although our inflammatory stratification approach, identified inflammatory clusters with varying grades of inflammation, an aggravated inflammatory response with marked elevations in peripheral cytokines were not evident in our study groups. In contrast, schizophrenia patients have previously shown elevated levels of inflammatory cytokines in peripheral and central tissues in association with lowered TSPO expression in the middle frontal gyrus (Coughlin et al., 2016; Notter et al., 2018a). However, this does not preclude that altered TSPO signalling could be the result of other underlying factors. These include systemic changes through interplay between various other cell types involved in neuroimmune modulation (Notter et al., 2018a) or a consequence of the oxidative stress during inflammation (Notter et al., 2018b), cellular metabolism changes and/or mitochondrial dysfunction (Gut, 2015; Batoko et al., 2015; Banati et al., 2014), all of which have been previously implicated in schizophrenia (Steullet et al., 2016).

Although, we do not present data in a confirmatory cohort as it is not customary with PET studies, longitudinal clinical follow-up revealed that 5 of the 39 individuals at CHR in this study (~13%) converted to psychosis. Although this was a cross-sectional study and conclusions were limited by small sample size, we found no significant differences in the levels of central or peripheral inflammatory markers (data not shown) between individuals at CHR who converted to psychosis and non-converters. More comprehensive indices such as proteomic prediction models may be better suited to predict transition to psychosis in CHR individuals over single inflammatory markers (Mongan et al., 2021). Together, our findings showing no evidence of altered brain TSPO levels among the inflammatory clusters (entire cohort or CHR) suggests that the negative relationship observed between TSPO expression and low-grade inflammation in schizophrenia is not evident in the putative prodromal state. Nevertheless, inflammatory stratification as depicted in the current study as opposed to most previous psychosis studies that focused on a singular marker at a time is crucial to understand the entangled interplay between multiple inflammatory signalling systems (central and periphery) in the body and across the psychosis spectrum.

Our exploratory analyses did not find any significant relationship between inflammatory clusters and symptom severity or cognition (entire cohort or CHR). This is in line with a previous study (Karanikas et al., 2017) which did not observe any relationship between clinical

symptom severity and cytokine levels in first-episode psychosis. However, two previous studies (Stojanovic et al., 2014; Zeni-Graiff et al., 2016) reported an association between peripheral cytokines' levels and severity of symptoms in line with the NAPLS evidence in CHR (Perkins et al., 2015).

On the contrary, CRP, IL-1β, TNF-α and IFN-γ levels in serum were found to independently predict TSPO in the brain (combined cohort). We found that the variance inflation factor (VIF) using regression analysis predicted only a moderate correlation among the significant predictors of brain TSPO (CRP: VIF = 1.01; IL-1β: VIF = 2.05; TNF-α: VIF = 1.00; IFN-γ: VIF = 2.06). Hence, the modelling takes care of the collinearity between the inflammatory proteins included in the model. Thus, the listed peripheral inflammatory markers are indeed reliable estimates of brain TSPO expression.

Coughlin and colleagues (Coughlin et al., 2016) reported an elevation of IL-6 levels in plasma of schizophrenia, however, found no association with TSPO. In contrast, LPS challenge produced significant TSPO elevation in the whole brain indicating a neuroimmune response associated with microglial activation in healthy adult brains (Woodcock et al., 2021). Interestingly, a longitudinal PET study in healthy male volunteers with repeated LPS challenges, found an initial increase and then a decrease in TSPO binding following subsequent LPS challenge (Nettis et al., 2020), suggestive of cerebral immunotolerance. Importantly, to date *in vivo* PET studies including patients with mood disorders found elevated brain TSPO levels compared with healthy subjects but no significant correlation between peripheral and central inflammation (Schubert et al., 2021). Recently, Attwells and colleagues (Attwells et al., 2020) reported that the natural logarithm of serum PGE$_2$/CRP and TNF$_\alpha$/CRP to be highly predictive of TSPO in mood or anxiety disorders.

Of interest, our inflammatory clusters (entire cohort or CHR) did not include CRP levels. This discrepancy between CRP results and other pro-inflammatory markers could be due to methodological differences. A high sensitivity ELISA was used to measure peripheral CRP levels in our study, while a multiplex assay with the high sensitivity T-cell panel was used to measure other interleukins which is considered more accurate than most cytokine ELISAs, especially at low concentrations. Additionally, IL-8 levels which was differentially expressed between study groups did not influence the cluster separation. The precise mechanism underlying this heterogeneity and the relevance of interplay among inflammatory markers (individual or combined) characterising low-grade inflammation in schizophrenia (Notter et al., 2018a) and its putative prodrome remains to be determined.

There are limitations in the interpretation of our findings. First, several types of cells in the brain including microglia, astrocytes, neurons, and vascular endothelial cells express TSPO (Notter et al., 2018a; Lavisse et al., 2012). However, these other cell types are also involved in the immune response, and thus the overall conclusion of this study remains unaffected. Second, while our sample sizes are relatively large for PET studies, the study may have been underpowered to test relationships between brain TSPO and peripheral biomarkers. However, all our main findings survived corrections for multiple comparisons. Also, based on our previous studies (Hafizi et al., 2017b; Da Silva et al., 2019) with similar sample sizes, we should have been well powered to test TSPO levels between inflammatory clusters. Third, while several studies have reported cytokine profiles in the peripheral blood of schizophrenia patients, only a few studies have compared cytokine profiles in CSF and plasma. One such study found comparatively elevated IL-6 CSF levels in patients with schizophrenia and major depressive disorder when compared to their serum levels (Sasayama et al., 2013). CSF measures may be of more clinical relevance given their proximity to brain tissue reflecting inflammatory abnormalities in brain tissue more closely compared to peripheral blood. To date, only one study in schizophrenia compared plasma and CSF measures and investigated their relationship with brain TSPO, however, found no relationship with brain TSPO but found increased CSF IL-6 levels that significantly correlated with their plasma levels (Coughlin et al., 2016). To our knowledge, no previous study has reported CSF levels in CHR. Lastly, the present study provides cytokine levels for only one snapshot in time; longitudinal studies are warranted to study cytokine dysregulations in the disease course.

5. Conclusion

Our well-characterized examination of inflammatory (including IL-2 and IL-10) clusters showed no evidence of altered brain TSPO levels among inflammatory clusters, highlighting the complexity of brain/periphery immune interactions. In conclusion, alterations in brain TSPO expression in response to low-grade inflammatory processes are not evident in CHR, but elevations in individual markers (IL-8) may relate to the severity of prodromal symptoms, particularly general symptoms.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100636.

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